

Patent  
Orrick No. 702015.5  
(Former L&L 265/130)

SEPHAROSE®, SEPHADEX®, TRISACRYL®, TSK-GEL SW OR PW®, SUPERDEX®, TOYOPEARL HW and SEPHACRYL®, ~~Source, Poros, Sepharose, sephadex, Trisacryl, TSK-gel SW or PW, Superdex, Toyoparl HW and sephaeryl,~~ for example, which are suitable for the application of this invention.

On page 7, lines 13-27, please replace with the following:

Examples of supports functionalised with a quaternary amine include the gels SOURCEQ, MONO Q, Q SEPHAROSE®, POROS® HQ and POROS® QE, FRACTOGEL® TMAE ~~Source Q, Mono Q, Q Sepharose, Poros HQ and Poros QE, Fractogel TMAE~~ type gels and TOYOPEARL SUPER® Q Toyoparl Super Q gels.

A particularly preferred support to perform the anion exchange chromatography comprises poly(styrene-divinylbenzene). An example of this type of gel which may be used within the scope of this invention is SOURCE ~~Source~~ Q gel, particularly SOURCE ~~Source~~ 15 Q (Ppharmacia). This support offers the advantage of very large internal pores, thus offering low resistance to the circulation of liquid through the gel, while enabling rapid diffusion of the exosomes to the functional groups, which are particularly important parameters for exosomes given their size.

On page 9, lines 15-22, please replace with the following:

To perform the gel permeation chromatography step, a support selected from silica, acrylamide, agarose, dextran, ethylene glycol-methacrylate co-polymer or mixtures thereof, e.g., agarose-dextran mixtures, are preferably used. As an illustration, for gel permeation chromatography, a support such as SUPERDEX® 200HR ~~Superdex-200HR~~ (Pharmacia), TSK G6000 (TosoHaas) or SEPHACRYL® S ~~Sephaeryl-S~~ (Pharmacia) is preferably used.

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On page 13, lines 15 through page 14, line 6, please replace with the following:

The affinity chromatography step can be performed in various ways, using different chromatographic support and material. It is advantageously a non-specific affinity chromatography, aimed at retaining (i.e., binding) certain contaminants present within the solution, without retaining the objects of interest (i.e., the exosomes). It is therefore a negative selection. Preferably, an affinity chromatography on a dye is used, allowing the elimination (i.e., the retention) of contaminants such as proteins and enzymes, for instance albumin, kinases, deshydrogenases, clotting factors, interferons, lipoproteins, or also co-factors, etc. More preferably, the support used for this chromatography step is a support as used for the ion exchange chromatography, functionalised with a dye. As specific example, the dye may be selected from Blue SEPHAROSE® Sepharose (Pharmacia), YELLOW 86, GREEN 5 and BROWN 10 ~~Yellow 86, Green 5 and brown 10~~ (Sigma). The support is more preferably agarose. It should be understood that any other support and/or dye or reactive group allowing the retention (binding) of contaminants from the treated biological sample can be used in the instant invention.

On page 15, lines 11-14, please replace with the following:

In another preferred embodiment, step b) above comprises a clarification of the culture supernatant, followed by an affinity chromatography on dye, preferably on Blue SEPHAROSE® ~~sepharese~~.

On page 17, lines 4-24, please replace with the following:

The results given in the examples demonstrate that the injection of an exosome preparation into the chromatography column allows to obtain symmetric absorption peaks, which are perfectly

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resolved (Fig. No. 1). The different fractions isolated in this way may be analysed using conventional protein electrophoresis techniques in denaturing gel followed by COOMASSIE® Coomassie blue staining techniques or specific protein detection techniques using antibodies. It is thus possible to demonstrate, for texosomes, that the peak eluted in anion exchange chromatography with a 400 mM saline solution has an identical protein profile to that of an exosome preparation prepared using conventional methods (Fig. No. 2). In fact, this allows to characterise the peaks eluted at lower or higher saline concentrations as related to distinct, biological contaminants. In another experiment, membrane vesicles produced from dendritic cells (dexosomes) or certain texosomes are eluted at a ionic strength comprised between about 500 and 700 mM, and mastocyte-derived vesicles at about 350 mM.

On page 25, lines 1-4, please replace with the following:

Figure No. 2: Protein profile analysis, by electrophoresis in SDS PAGE followed by COOMASSIE® Coomassie blue staining, of the different elution fractions of an exosome preparation.

On page 25, lines 10-26, please replace with the following:

Figure No. 5: General profile of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow step, following 600 and 10 000 g centrifugations of a dexosome supernatant.

Figure No. 6: Western blot against MHCII molecules of the exosomes, in the non-adsorbed fraction and the eluate of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow step.

Figure No. 7: General profile of the SOURCE Source Q15 step following a Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow step.

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Figure No. 8: Detail of the elution gradient on the SOURCE Seuree Q15 following a Blue SEPHAROSE® ~~Sepharese 6 FAST FLOW 6 fast-flow~~ step (enlargement from bottom right of fig 7).

Figure No. 9: Purification profile of exosomes produced by RBL DR+ cell line (eq. 53µg proteins) and purified on SOURCE Seuree Q15 column after Dnase and Rnase treatment.

Figure No. 10: Separation of exosomes by a discontinued NaCl gradient on a SOURCE Seuree Q15 support.

On page 26, lines 16-21, please replace with the following:

2) SDS PAGE protein analysis

20 µl of sample is diluted in Laemmli buffer (Nature 227 (1970) p.680-685) and then subjected to thermal denaturation at 95°C for 10 min and then loaded on 10% acrylamide gels (Novex 1 mm x 10 wells). After migration, the gels are stained with COOMASSIE® Coomassie blue.

On page 27, line 27 through page 28, line 12, please replace with the following:

40 µg of this exosome preparation diluted in 500 µl of pH 8 50 mM HCl/Tris buffer is injected onto a column containing SOURCE Seuree Q 15 gel (Pharmacia) stabilised in a pH 8 50 mM HCl/Tris buffer. After rinsing, the adsorbed species are eluted on 30 volumes of column with a linear 0 to 500 mM NaCl gradient, followed by a 2 M NaCl solution. The elution fractions are analysed by spectrophotometry at 260 and 280 nm. The elution fractions are grouped into 5 major fractions (F1 to F5), to analyse their respective protein profile. The proteins of each fraction are precipitated with 1/10 volume of a 100% trichloroacetic acid solution and then rinsed with an acetone solution. The protein pellets are taken up with 20 µl of Laemmli solution and are deposited on an SDS PAGE acrylamide gel which is then stained with COOMASSIE® Coomassie blue.

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On page 30, line 21 through page 31, line 27, please replace with the following:

. Buffers and stock solutions

0.22  $\mu$ m filtered stock solutions are used, except for the water and soda solution. The first buffer is a 100 mM (Sigma, 99% purity) Bis-Tris-Propane (BTP) solution, buffered to pH 6; this buffer is connected to channel A of the chromatograph (BioCad Sprint, Perkin-Elmer). The second buffer is a 100 mM Bis-Tris-Propane solution, buffered to pH 9; this buffer is connected to channel B of the chromatograph. The water is produced on resin with a MILLI-Q™ Milli-Q system (Millipore) at a resistance of 18 M $\Omega$ cm. The water is connected to channel C. A 3 M sodium chloride stock solution (NaCl, Prolabo, 99.5% purity) is connected to channel D. A 0.1 M soda solution (NaOH, Prolabo, 98% purity minimum) is connected to channel F. Channel E is used to load the culture supernatants onto the columns.

All the buffers are produced from water produced by the MILLI-Q™ Milli-Q system and are not degassed.

. Columns

Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow (Pharmacia):

The first step is performed with a Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow (Pharmacia) column. The matrix is agarose linked with Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow Blue 3G (7%). The particle size is between 45 and 165  $\mu$ m. The maximum linear flow rate is 750 cm/hour. The gel is stable at pH values between 4 and 12; at the extreme pH values, 4 and 12, the gel may be damaged, inducing a decrease in the fixing capacity (segregation of the

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Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow and an increase in pressure (fine formation).

Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow is specific for compounds such as albumin, kinases, dehydrogenases and other enzymes containing cofactors such as NAD<sup>+</sup>, clotting factors, interferons and lipoproteins.

The theoretical fixing capacity of Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow is approximately 15 to 20 mg of serum albumin per ml of gel.

On page 32, lines 5-17, please replace with the following:

SOURCE Source 15Q:

The second step is performed with a SOURCE Source 15Q (Pharmacia) column, a strong anion exchanger. The matrix is polystyrene cross-linked with divinyl benzene. The bead size is 15 µm and homogeneous. The beads are passed through a system of pores with a size varying from 20 to 1000 nm. These gels are very resistant to pressure and withstand high linear flow rates (1800 cm/hour and over), while retaining a satisfactory resolution and capacity. This is made possible by the homogeneity of the beads and their porosity which increases the access of the molecules to the functional groups.

On page 32, lines 21-30, please replace with the following:

The theoretical fixing capacity of this exchanger is approximately 25 mg of proteins per ml of gel. A 0.8 ml column (PEEK column 4.6 mm ID/50 mm L, Perkin-Elmer) is used, giving a maximum capacity of 20 mg of proteins. The actual capacity, ensuring a good resolution, is approximately 10% of this maximum capacity, or 2 mg of proteins. The flow rate used is 5 ml/min

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(1880 cm/hour) and allows quick separations. The column is packed with the POROS@SELF PACK SYSTEM ~~Poros-selfPack-system~~ at 15 ml/min at 150 bar.

On page 33, lines 1-8, please replace with the following:

. Chromatograph (BIOCAD SPRINT ~~BioCad Sprint~~)

The BIOCAD BioCad is an HPLC (High Performance Liquid Chromatography) system which makes it possible to work at high pressures (maximum bar) and at flow rates ranging from 0.2 to 60 ml/min. Up to 6 buffers may be connected (the 6 channels are currently used) and the system may be treated with 0.1 M soda (pH 12) to depyrogenate the tubing and column.

On page 33, lines 23-26, please replace with the following:

Finally, the separated sample is either recovered in a 50 ml FALCON TUBE ~~Falcon tube~~ or collected in siliconed EPENDORF ~~Eppendorf~~ tubes (ADVANTEC ~~Advantec~~ SF-2120 collector) to minimise non-specific interactions.

On page 34, lines 6-10, please replace with the following:

4.2) Blue SEPHAROSE@ ~~Sepharese~~ 6 FAST FLOW ~~6 fast flow~~ stage

The culture supernatants are centrifuged twice at 600 g and once at 10,000 g before being loaded onto the Blue SEPHAROSE@ ~~Sepharese~~ 6 FAST FLOW ~~6 fast flow~~ column.

On page 34, line 25 through page 35, line 7, please replace with the following:

Quantitative and qualitative aspect of the Blue SEPHAROSE@ ~~Sepharese~~ 6 FAST FLOW ~~6 fast flow~~ stage.

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As described above, the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow stage is specific for proteins such as albumin which is a major contaminant of the culture supernatant. The protein concentration of each fraction of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow stage is measured using a BIORAD Biorad technique (OD measurement at 600 nm). The results are given in table 1.

Table 1. Protein concentration in each fraction of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow stage.

On page 35, line 10 through page 36, line 15, please replace with the following:

Most of the proteins are detected either in the eluate (72%) or in the regeneration (32%). The non-adsorbed fraction only represents 7 to 10% of the total proteins loaded onto the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow column. The stage is specific for the major contaminants of the supernatant. To check this specificity of Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow, each fraction is deposited on an SDS-PAGE gel under reducing conditions and stained with silver nitrate. The overload of the column (50 ml supernatant loaded onto a 5.5 ml Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow column) was used to calculate the maximum quantity of proteins of the culture supernatant that it is possible to load onto the column per ml of gel (table 2). In this case, the percentage of the non-adsorbed fraction increases from 7 to over 30% of the quantity of protein loaded. This value is between 16 and 18 mg of proteins per ml of Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow gel. In conclusion, 1 ml of Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast flow gel allows the purification of approximately 5

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to 6 ml of culture supernatant (2.5% HSA AIMV). This value is very important for the scale-up study since it determines the size of the column to use and, consequently, the cost of this stage.

Table 2. Overload study of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow stationary phase in the first stage.

On page 36, line 18 through page 37, line 19, please replace with the following:

As expected, the major contaminant of the culture supernatants, after the 600 and 10,000 g centrifugations, is albumin. After fixing on the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow, this contaminant is detected in the eluate and regeneration fractions. In addition to this contaminant, many other low and high molecular weight contaminants are detected.

The Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow stage enables the elimination of approximately 90 to 95% of the culture supernatant contaminants. The exosomes are found in the non-adsorbed fraction of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow (fig. 6).

4.3) Anion exchanger step: SOURCE source 15Q (Pharmacia).

A 0.8 ml SOURCE source 15Q column is used with a 5 ml/min flow rate (linear flow rate 1880 cm/hour, contact time 0.1 min) with a pressure of approximately 50 bar. The non-adsorbed fraction of the Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow is loaded directly onto the SOURCE source 15Q (fig. 7) with no modifications in the saline (NaCl) concentration or the pH.

On page 38, lines 9-19, please replace with the following:

Quantitative and qualitative aspects of the SOURCE source 15Q stage.

As illustrated in figure 6, most of the proteins of the non-adsorbed Blue SEPHAROSE® Sepharose 6 FAST FLOW 6-fast-flow fraction are not retained on the column. The protein

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concentrations were measured using a BIORAD Biorad test (OD absorption at 600 nm) and are summarised in table 3.

Table 3. Quantitative aspects of the SOURCE Source 15Q stage after a Blue SEPHAROSE® ~~Sepharese~~ 6 FAST FLOW 6-fast-flow stage.

On page 39, lines 2-16, please replace with the following:

95% of the proteins loaded on the SOURCE Source 15Q column are not retained. The pooled fractions 4 to 6 and the pooled fractions 19 to 23 represent approximately 1.5%. The regeneration was not assayed. The yield is very close to 100%: all the loaded proteins and vesicles are eluted from the column. In terms of the column's protein concentration, SOURCE Source 15Q is capable of binding approximately 25 mg of proteins (manufacturer's data), corresponding to 20 mg of proteins for 0.8 ml columns. It is clear that these values are very far from the column's maximum and the 5 to 10% for a good resolution, since they range between 1 and 2 mg of proteins. Under these conditions, with a 0.8 ml SOURCE Source 15Q column, it is possible to purify between 200 and 400 ml of culture supernatant.

On page 40, lines 15-27, please replace with the following:

In two chromatography stages, the first involving a negative selection (Blue SEPHAROSE® ~~Sepharese~~ 6 FAST FLOW 6-fast-flow stage) of exosomes and the second involving a positive selection and a selective elution of exosomes (SOURCE Source 15Q stage), approximately between 99 and 99.5% of the proteins in the culture supernatant are eliminated, while retaining the exosomes. The process preserves the integrity of the exosomes in electron microscopy. Therefore, this process is specific for exosome purification.

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As an example, other columns retaining serum proteins may be used. In addition, cationic macroporous columns may be used instead of SOURCE Seuree 15Q.

On page 41, line 14 through page 42, line 2, please replace with the following:

The ultracentrifugation sediment is loaded onto an ion exchange column, SOURCE Seuree 15Q, (Pharmacia) (fig. 9). A 0.8 ml column is used with a 5 ml/min flow rate (linear flow rate 1880 cm/hour, contact time 0.1 min).

The column is equilibrated in 12 mM BTP buffer (150 mM NaCl, pH 7). After loading the sample, the column is washed with 15 to 20 column volumes of the same equilibrating buffer. The elution is performed with 25 column volumes by varying the saline concentration from 150 mM to 1 M NaCl, the pH is kept constant (fig. 9). The protein contamination of the medium is very low since the cells are washed in PBS and the induction and release of the exosomes are performed in RPMI only. This is confirmed by the absorption observed (280 and 254 nm) in the non-adsorbed fraction of SOURCE Seuree 15Q. The presence of the exosomes is confirmed by a Western Blot analysis targeted against MHC II molecules (fig. 11) after separation of the peaks with steps in NaCl (fig. 10) and ultracentrifugation of each of the peaks.

On page 43, lines 2-11, please replace with the following:

SOURCE Seuree 15Q is able to retain exosomes and separate them from potential contaminants (fig. 9). The vast majority of the exosomes are eluted in the same peak at 350 mM NaCl. In electron microscopy, the exosomes appear "normal" with a specific label for human MHC II molecules. The heterogeneity of the size of the exosomes (peak 1) appears to indicate that the separation is based on ion exchanges and not on screening. Therefore, SOURCE Seuree 15Q may be used as a purification step for RBL exosomes.